Enzyme and Microbial Technology 69 (2015) 1–9

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Characterization of glutathione *S*-transferases from *Sus scrofa*, *Cydia pomonella* and *Triticum aestivum*: Their responses to cantharidin

Xue-Qing Yang [a,b], Ya-Lin Zhang [a,*]

[a] *Key Laboratory of Plant Protection Resources & Pest Management of the Ministry of Education, College of Plant Protection, Northwest A & F University, Yangling, Shaanxi 712100, China*
[b] *College of Plant Protection, Shenyang Agricultural University, Shenyang 110866, Liaoning, China*

ARTICLE INFO

Article history:
Received 15 September 2014
Received in revised form 6 November 2014
Accepted 14 November 2014
Available online 24 November 2014

Keywords:
Glutathione *S*-transferases
Cantharidin
Stress
Biochemical characterization
Binding protein

ABSTRACT

Glutathione *S*-transferases (GSTs) play a key role in detoxification of xenobiotics in organisms. However, their other functions, especially response to the natural toxin cantharidin produced by beetles in the Meloidae and Oedemeridae families, are less known. We obtained GST cDNAs from three sources: *Cydia pomonella* (*CpGSTd1*), *Sus scrofa* (*SsGSTα1*), and *Triticum aestivum* (*TaGSTf3*). The predicted molecular mass is 24.19, 25.28 and 24.49 kDa, respectively. These proteins contain typical N-terminal and C-terminal domains. Recombinant GSTs were heterologously expressed in *Escherichia coli* as soluble fusion proteins. Their optimal activities are exhibited at pH 7.0–7.5 at 30 °C. Activity of CpGSTd1 is strongly inhibited by cantharidin and cantharidic acid, but is only slightly suppressed by the demethylated analog of cantharidin and cantharidic acid. Enzymatic assays revealed that cantharidin has no effect on SsGSTα1 activity, while it significantly stimulates TaGSTf3 activity, with an EC_{50} value of 0.3852 mM. Activities of these proteins are potently inhibited by the known GST competitive inhibitor: *S*-hexylglutathione (GTX). Our results suggest that these GSTs from different sources share similar structural and biochemical characteristics. Our results also suggest that CpGSTd1 might act as a binding protein with cantharidin and its analogs.

1. Introduction

Mammals, insects, and plants are continuously exposed to xenobiotics, compounds that may cause toxic and sometimes lethal effects [1]. The naturally occurring toxicant cantharidin, the active ingredient of cantharides, is predominantly produced by *Cantharis vesicatoria* [2]. Cantharidin was recently reported to possess insecticidal toxicity [2–4] and its emulsifiable concentrate (EC) formulation has been developed into a bio-pesticide. Apart from its insecticidal activity, endothall, one of the cantharidin analogs, has been developed into a commercial herbicide that inhibits the protein phosphatase 2A (PP2A) activity in plants [5,6]. Cantharidin and its analog residues may interact deleteriously with mammals during feeding. To survive the effects of these naturally occurring xenobiotic compounds, organisms have evolved intricate biological adaptation mechanisms [7].

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are dimeric proteins existing in both prokaryotic and eukaryotic organisms involved in cellular detoxification. They catalyze the conjugation of glutathione (GSH) with a wide range of endogenous and exogenous agents, including carcinogens, therapeutic drugs, pesticides, environmental toxins and products of oxidative stress [8–10]. GSTs are also responsible for regulating important cell signaling pathways and biosynthesis [10]. To date, there are three categories of GSTs: cytosolic, microsomal and mitochondrial [10,11]. Mammal cytosolic GSTs are further divided into seven classes: alpha, mu, pi, sigma, omega, zeta and theta [10]. The class alpha contains many possible isoenzymes with different activities, tissue distribution and substrate specificities. Insect cytosolic GSTs are divided into six classes: delta, epsilon, omega, sigma, theta, and zeta [12]. The delta and epsilon classes are arthropod-specific GSTs involved in the detoxification of xenobiotics [9,13]. Plant cytosolic GSTs are grouped into seven classes: theta, zeta, phi, tau, lambda, glutathione-dependant dehydroascorbate reductase (DHAR), and tetrachlorohydroquinone dehalogenase [14]. The phi and tau GST subfamilies are responsible for herbicide detoxification [15].

Many GSTs have been isolated and characterized from bacteria, plants, insects, fish and mammals [8]. Recent studies suggest that cantharidin possesses insecticidal toxicity and inhibits the activity of insect GSTs [2–4]. However, knowledge about the

* Corresponding author. Tel.: +86 29 87092190; fax: +86 29 87092190.
E-mail address: yalinzh@nwsuaf.edu.cn (Y.-L. Zhang).

http://dx.doi.org/10.1016/j.enzmictec.2014.11.003
0141-0229/

Table 1
PCR primers used in this study.

Name	Sequence (5′–3′)	Primer used
SsGSTα1F	C***GGATCC*** ATGGCGGGGAAGCCCATTC	Cloning SsGSTα1 ORF
SsGSTα1R	T***AAGCTT***TTAATCCGGAAAATATTCTTG	
CpGSTd1F	C***GGATCC***ATGCCAGACCTATACTACG	Cloning CpGSTd1 ORF
CpGSTd1R	T***AAGCTT***TTACTTCTTCATCATGCTG	
TaGSTf3F	C***GGATCC***ATGGCGCCGGCGGTGAAGG	Cloning TaGSTf3 ORF
TaGSTf3R	T***AAGCTT***CTACTCTGCTTTCTTTCCAAG	

The start codon and stop codon are underlined. The *Bam*H I and *Hin*d III restriction enzyme sites are in italicized bold.

effect of cantharidin on GSTs from other organisms, especially mammals and plants is poorly known. In this study, we identified different classes of GST from three sources, including alpha class GST *Sus scrofa* (SsGSTα1, GenBank accession no. NM_214389.1), delta class GST from *Cydia pomonella* (CpGSTd1, GenBank accession no. EU887533.1), and phi class GST from *Triticum aestivum* (TaGSTf3, GenBank accession no. AJ440792.1), representing mammals, insects, and plants, respectively. The recombinant biochemical properties, sensitivity to inhibitors and response to cantharidin and its analogs of recombinant GST proteins were investigated.

2. Experiment

2.1. Materials

Escherichia coli strains DH5α and BL21 (DE3) were used as host cells and were cultivated according to the instructions of the suppliers (Takara, Dalian, China). The restriction enzyme and *Taq* polymerase were also obtained from Takara (Takara). Cantharidin and norcantharidin were obtained from Alfa Aesar Chemical Co. Ltd. (Haverhill, MA, USA). Their analogs, cantharidic acid and norcantharidic acid (purity > 95%) were previously synthesized [16]. The GST inhibitor [17] *S*-hexylglutathione (GTX) was purchased from Aladdin Reagent (Shanghai, China). The 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione were purchased from ABCR GmbH & CO. KG (Germany) and Sigma–Aldrich Life Sciences (St. Louis, MO), respectively. Other chemicals and reagents were commercially available. The cDNAs from *S. scrofa* liver and *T. aestivum* lamina were kindly supplied by the College of Animal Science and Technology, and the State Key Laboratory of Crop Stress Biology for Arid Areas (Northwest A & F University, China), respectively.

2.2. Sequence analysis of GSTs

The amino acid sequences of *SsGSTα1*, *CpGSTd1* and *TaGSTf3* were deduced and the theoretical isoelectric point (*pI*) of the deduced proteins were predicted using ExPASy Proteomics [18]. The amino acid sequence of SsGSTα1, CpGSTd1 and TaGSTf3 were aligned using Clustal W2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). A similarity analysis of SsGSTα1, CpGSTd1 and TaGSTf3 was performed using the BLAST web program (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The secondary structure of these proteins was predicted using PSIPRED [19]. Phylogenetic analyses were constructed by using MEGA4 [20] using the neighbor-joining method with 1000 bootstrap replicates.

2.3. GSTs isolation and construction of the expression plasmid

The total RNA of *C. pomonella* was extracted from five third instar larvae using the RNAiso Plus Kit (Takara) according to the manufacturer's instructions. The RNA was then digested with DNase I (MBI, Fermentas) to remove the genomic DNA. Subsequently, the first strand cDNA was synthesized from 1 μg of total RNA using the RevertAid™ First Strand cDNA synthesis kit (MBI) as described by the manufacturer. The ORF of *SsGSTα1*, *CpGSTd1* and *TaGSTf3* were amplified using the following primer pairs: SsGSTα1F and SsGSTα1R, CpGSTd1F and CpGSTd1R, and TaGSTf3F and TaGSTf3R, respectively. These primers were introduced into *Bam*H I and *Hin*d III restriction enzyme sites as the forward and reverse primer (Table 1), respectively. PCR was performed on a C1000 Thermal Cycler (BioRad, USA) using high-fidelity Ex *Taq* polymerase (Takara) to eliminate any potential error occurring with *Taq* DNA polymerase. The PCR product was gel purified using the Biospin Gel Extraction Kit (Bioer Technology Co., Ltd, China) and cloned into pMD-19T vector (Takara), and then transferred into *E. coli* DH5α (Takara). The sequenced plasmid (Shanghai Sunny Biotech Co., Ltd, China) was digested by *Bam*H I and *Hin*d III, and thereafter cloned into the expression vector pET-32a (+) and transformed into the host strain, *E. coli* BL21(DE3).

2.4. Expression and purification of recombinant GSTs

The positive *E. coli* colonies were grown in 400 ml of Luria-Bertani (LB) medium containing 100 μg ml^{-1} ampicillin. After OD_{600} reached 0.6, the recombinant GST expression was induced with a 0.2 mM final concentration of isopropyl β-D-thiogalactopyranoside (IPTG) at 25 °C with shaking at 220 rpm for 12 h.

Bacterial cells were harvested by centrifugation at 8000 × *g* for 10 min. The pellets were re-suspended in lysis buffer (10 mM Tris–HCl, 1 mg ml^{-1} lysozyme, pH 8.0) and incubated at room temperature for 30 min. After sonication for 5 min on ice, the supernatant was separated by centrifugation at 12,000 × *g* for 30 min at 4 °C. The soluble fraction was loaded onto a Ni^{2+}-NTA agarose gel column (TRANS, China) and recombinant protein was purified [21]. Eluted fractions were analyzed on a 12% SDS-PAGE gel (BioRad, USA). The target protein was dialyzed against sodium phosphate buffer (50 mM, pH 7.2) overnight to desalt. The concentration of purified protein was quantified by the Bradford method [22].

2.5. Enzyme kinetic properties of purified GSTs

The kinetic constants of purified recombinant GSTs were determined using 0.01–1.6 mM of CDNB (with GSH held constant at 10 mM), or 0.0625–4 mM of GSH (with CDNB held constant at 2 mM) in 50 mM sodium phosphate buffer (pH 7.2) at 30 °C. GST activity was measured as described previously [11]. The difference in absorbance value at time 1 min (t_1) and at time zero (t_0) was used to calculate activity. Results were expressed as nM glutathione conjugated mg of protein^{-1} min^{-1} (ε = 9.6 mM^{-1} cm^{-1}). The protein replaced by sodium phosphate buffer was used as the control. Each test was conducted in triplicate. The rate of reaction was used to construct a double-reciprocal Lineweaver–Burk plot of 1/*V versus* 1/*S*, and the kinetic constants, Michaelis constants (K_m) and V_{max} were determined.

2.6. Effect of pH and temperature on GSTs activities

Effects of pH on the activities of SsGSTα1, CpGSTd1 and TaGSTf3 were determined by using a varying pH (4.0–9.0) with sodium phosphate buffer (50 mM). The effects of temperature on the activities of SsGSTα1, CpGSTd1 and TaGSTf3 were measured by performing the reaction at temperatures ranging from 15 to 45 °C.

2.7. Effects of cantharidin and its analogs on GSTs activities

To investigate the effects of cantharidin and its analogs on GSTs activities, the purified CpGSTd1 was pre-incubated under final concentrations of 4 mM cantharidin, norcantharidin, cantharidic acid and norcantharidic acid, respectively, for 5 min at 30 °C before the addition of CDNB. Remaining activity was measured and CpGSTd1 replaced by sodium phosphate buffer was used as the control.

The effects under different concentrations of cantharidin on SsGSTα1, CpGSTd1 and TaGSTf3 activity were determined by pre-incubating 2 μl protein with 2, 0.2, and 0.02 mM cantharidin for 5 min. The reaction then was initiated by adding CDNB. The remaining activity was measured. For the positive control, the GTX was used and its half-inhibitory concentrations (IC_{50}) on SsGSTα1, CpGSTd1 and TaGSTf3 were determined.

2.8. Structural modeling for GSTs

3D structure of SsGSTα1 and TaGSTf3 were predicted using homology modeling (automated mode) using the SWISS-MODEL (http://swissmodel.expasy.org). The target template sequence was searched using BLAST against the primary amino acid sequence contained in the SWISS-MODEL template library. A total of 157 templates were found. For each identified template, the template's quality was predicted from features of the target-template alignment. The templates with the highest quality were then selected for model building. Thus, a 1.80 Å resolution crystal structure of Glutathione *S*-transferase A3 from the human (PDB no. 2vcv.1.A) and a 2.80 Å resolution crystal structure of *Arabidopsis thaliana* (araGST) (PDB no. 1bye.1.B) were selected from the PDB database and used as the templates for SsGSTα1 and TaGSTf3, respectively.

2.9. Statistical analysis

Statistical analysis was conducted using SPSS 12.0 (IBM, Chicago, USA). The effects of cantharidin and its analogs on GSTs activities were statistically analyzed using Student's *t*-test ($^*P \le 0.05$; $^{**}P \le 0.01$; $^{***}P \le 0.001$). Results are shown as the mean of triplicates ± standard deviation (SD) and were plotted using GraphPad Prism 5 (San Diego, USA).

3. Results

3.1. Molecular and sequence analysis of GSTs

The open reading frame of *CpGSTd1*, *SsGSTα1* and *TaGSTf3* were 648, 669 and 669 bp, respectively, encoding CpGSTd1, SsGSTα1 and